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Potential Antibacterial Activity of Bioactive β-sitosterol from Root Bark of *Rhizophora apiculata* from Lampung Coastal

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Abstract

β-sitosterol is an essential bioactive phytosterol naturally present in plant cell membranes. It has a coincident structure with animal cholesterol. This investigation reported isolation, structure analysis, and an antimicrobial assay of β-sitosterol from the root bark of Bakau Minyak (*Rhizophora apiculata)* from Lampung coastal. The isolation of β-sitosterol was carried out through maceration using methanol, separation by vacuum liquid chromatography (VLC), and purification by column chromatography (CC) using ethyl acetate/n-hexane (2:8) as eluent. The structure of β-sitosterol was determined using spectroscopic analysis (UV-Vis, FT-IR, ^{13}C-NMR, ^{1}H-NMR, DEPT, and GC-MS). The pure β-sitosterol has 107.4 mg of white needle crystalline compound, the compound melting point about 140.7-141.2°C, the molecular mass confirmed by *m/z* 414, and UV absorption detected at λ 203.9 nm. The β-sitosterol antimicrobial bioactivity assay has shown potential activity to be developed as a lead compound against *E. coli.*

1. Introduction

β-sitosterol (stigmast-5-en-3β-ol) is a bioactive secondary metabolite type phytosterol naturally found in plant cell membrane surfaces, providing a chemical structure similar to that of mammalian cholesterol [1]. This compound is commonly found in various higher plant parts such as seed, wood, and root bark [2]. Bioactivity of β-sitosterol has been widely reported that it has antibacterial activity [3], non-alcoholic fatty liver disease (NAFLD) protecting agent [4], analgesic [5], anticancer [6], immunomodulatory [7], lipid-lowering effect [8], hepatoprotective [9], antidiabetic [10], respiratory protecting effect [11], wound recovery effect [10], antioxidant [12] and anti-inflammatory [13]. β-sitosterol is a potential micronutrient and has been confirmed by the Food and Drug Administration (FDA) to degrade blood cholesterol levels, reduce heart disease risk, and have no prominent side effects identified [14, 15].

Mangrove forests found in Indonesia's coastal areas exhibit habitats with high biodiversity of animals, plants, and microorganisms [11]. Mangroves have been reported to generate steroid, alkaloids, and terpenoids [16], which have biological benefits such as antidiabetic [17], antibacterial [18], anticancer [19], and antioxidant abilities [20, 21]. These bioactivity properties of mangroves have impressed pharmaceutical industry interest [22]. This plant has been used as traditional medicines to treat diarrhea and asthma [23], scabies treatment, and rheumatism [24]. *R. apiculate* or known as Bakau Minyak in Lampung (Figure 1), is common mangrove growing on the coastal zones of Lampung. This plant is hardy, tannin-rich, and has a high density, mainly used for construction and charcoal making [23].

Bakau Minyak has a high salt tolerance. These natural properties provide several bioactive compounds produced in response to environmental stresses. The root bark of Bakau Minyak, which part has direct interaction with seawater, is mainly reported containing tannins and steroids [25], indicating it has a potential source of β-sitosterol.

Figure 1. Bakau Minyak (*R. apiculata*) from Lempasing, South Lampung.

Although *R. apiculata* plays an essential role in traditional medicine in many tropical countries [11, 23], including Indonesia, only limited research has been scientifically reported to explore the bioactive and antimicrobial compound activity of this plant. Thus, the present study aims to investigate the antimicrobial activity of the bioactive β-*sitosterol* from the root bark of *R. apiculata* to increase the potential of mangrove forests on the Lampung coast. β-sitosterol was isolated using several chromatography techniques. Structural elucidation was carried out by spectroscopy analysis. The antimicrobial assay was determined by the agar diffusion method against *Escherichia coli*, *Bacillus subtilis*, and *Aspergillus niger*.

2. Methodology

The root bark of Bakau Minyak was obtained from the Research Center of Marine Development Cultivation (Balai Besar Pengembangan Budidaya Laut) Lempasing Coastal, Lampung, Indonesia. The specimen was determined as *R. apiculata* (Specimen number: IT IS.507389) by Botany Herbarium Bogoriensis in the Biology Research Center of The Indonesian Institute of Sciences (LIPI) Cibinong, West Java.

2.1. Equipment/ Tool/ Material

The chemicals used were ethyl acetate, methanol p.a. (Merck), n-hexane, acetone p.a. (Merck), 1.5% cerium sulfate/sulfuric acid (2 N), benzene p.a. (Sigma), chloroform p. a. (Merck), dichloromethane (Sigma), silica gel G 60 (Merck), silica gel 35-70 Mesh 60 (Merck), silica gel 60 F254 0.25 mm TLC plate (Merck), microbial medium growth: nutrient broth and nutrient agar (Supelco). Compound purification monitored using thin-layer chromatography was carried out on TLC plate PF_{254}, and the spots were figured under ultraviolet light (254 and 366 nm) and by exposure using cerium sulfate vapor. The methanol extracts were obtained by rotary evaporator HEI-VAP (Heidolph). The UV spectrum was obtained using UV-Vis (Shimadzu 1200). The melting point was measured using Melting Point Apparatus (Toledo HL3A). The β-sitosterol crystals were placed in a capillary glass tube, which was then inserted on the heating side. The crystals were heated until they melted, and the temperature was recorded. The IR spectra were obtained by FTIR spectroscopy (Scimitar 2100) using KBr pellets. The mass spectrum were acquired by GC-MS model QP2010 (Shimadzu) equipped with a VF5 column mass spectrometer (flow rate 1.8 mL/min) with diameter of 0.25 mm, length of 30 m, thickness of 0.25 µm and under 200°C ion source/ -70 eV at 40-650°C with a flow rate of 4°C/min (carrier gas was He). ^{13}C and ^{1}H NMR spectra were obtained by JEOL variant 500 MHz spectroscopy in acetone-d_6 and TMS as the zero-shift standards.

2.2. Extraction and Isolation

All extraction and isolation steps of β-sitosterol are shown in Figure 2. The dried powder of *R. apiculata* root bark (532 g) was extracted with methanol (4×2 L) under low pressure to obtain a final residue (58.7 g) of crude methanol extract (CME). The extraction occurred in about 72 hours with further removal of the solvent at a rotary evaporator at 50°C/120 rpm. CME was partitioned in a solvent to present hexane (HE) (17.2 g) and ethanol (21.4 g) extracts (EE). This study focused on the HE fractions because the EE fraction has high tannin content, making it more difficult to eliminate the tannin tint in the separation process [25]. 15.5 g HE was chromatographed using the vacuum liquid chromatography method, eluted using a gradient solvent by increasing the polarity of the eluent (hexane/ethyl acetate) to produce 33 fractions. The fractions 3, 4, and 5 were re-chromatographed using silica gel G-60 and eluted with hexane: ethyl acetate (4: 1, v/v). Among the 16 fractions obtained, fractions 3-9 were purified using a silica gel column to give a compound mixture (403.7 mg). The fraction of 403.7 mg was chromatographed on the silica gel column to produce 12 fractions. Fractions 4-9 yielded compound 1 (115.8 mg). After recrystallization in hexane, this fraction was identified as β-sitosterol (107.4 mg).

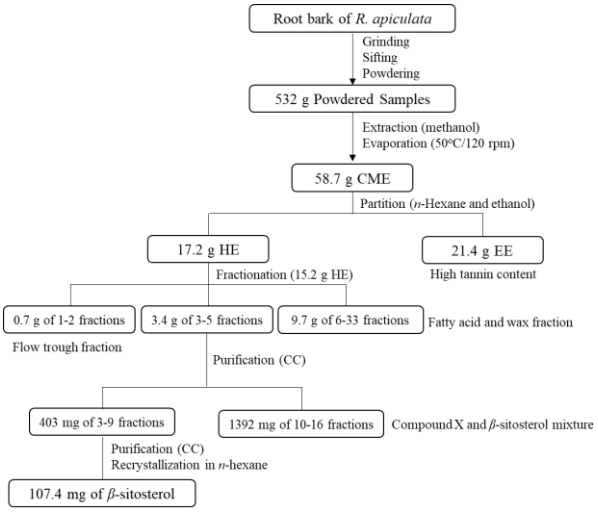

Figure 2. The flow chart of β-sitosterol isolation.

2.3. Antimicrobial Assay

The fungal and bacterial strains were acquired from the microbiology laboratory of The Indonesian Institute of Sciences LIPI-Cibinong and Biochemistry laboratory of the Chemistry department, University of Lampung collections. The antimicrobial assay was determined against *E. coli*, *B. subtilis*, and *A. niger* by the agar diffusion method. Both fungi and bacterial were cultured in nutrient broth (NB) medium with a conical flask serial, incubated at 37°C/24 h. Pre-warmed NB agar plates were seeded with 10^7-10^8 CFU suspension of bacteria and fungi. The paper discs (5 mm Whatman assay disc) were drilled into the wells plate, then the compound and the extract were slowly added until discs were filled. After all filled discs were placed in an upright position and then incubated at 37°C for 24 h, the inhibition zones were measured in millimeters (mm). The antimicrobial assay protocol was carried out under aseptic conditions, based on a standard procedure adapted from Balouiri *et al.* [26] with some modification.

3. Results and Discussion

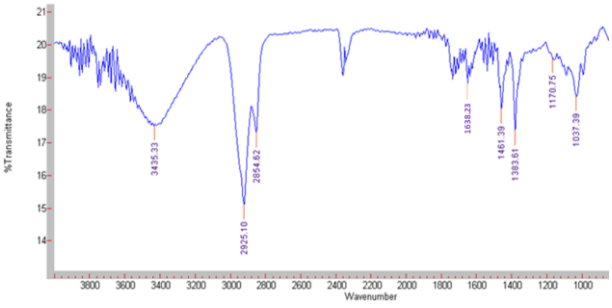

Figure 3. The IR spectrum of β-sitosterol

About 107.4 mg of pure white needle crystalline compound was isolated with the melting point of 140.7-141.2°C. The molecular mass was at *m/z* 414, confirmed by the GC-MS chromatogram, and the UV absorption peak was detected at λ of 203.9 nm in methanol. Based on Figure 3, the FTIR spectrum exhibits the absorptions band (cm^{-1}) for stretching O-H at 3435.33, CH_3 at 2925.10, CH_2 at 2854.62, and both absorptions C-H (sp^3) stretching for asymmetrical and symmetrical, respectively. Non-conjugated olefinic (C=C) at 1638.23, methylene in non-aromatic cyclic groups $(CH_2)_n$ at 1461.39, C-H bending is for scissoring and rocking of the germinal dimethyl group at 1382.09 and C-OH from secondary alcohol at 1037.39. The FTIR spectrum analysis has detected the nature of oxygen identified as a hydroxyl group. The FTIR spectrum also detects the existence of one C=C in the structure. The FTIR data are summarized in Table 1.

The NMR spectrum (Figure. 4) exhibits the existence of fifty protons containing six methyl (CH_3), eleven methylene (CH_2), nine methines (CH), and one hydroxy group. The presence of two singlets at δ 0.84 and 0.83 ppm confirm the existence of two CH_3 attached to the quaternary carbons. The appearance of two multiplet signals at δ 2.3 ppm indicates that two methylene protons are adjacent to the carbon attached to the hydroxyl group. A proton signal at δ 3.52 ppm in multiple properties presents a proton connected to the carbon, which is correlated with the OH groups. The overlapping triplets at δ 5.38 ppm are assigned to the olefinic protons.

Table 1. The IR spectrum of β-sitosterol

Absorption band (cm^{-1})	Functional Groups
3435.33	O-H stretching
2925.10	C-H (sp^3) stretching
2854.62	C-H (sp^3) stretching
1638.23	C=C (cyclic alkene) stretching
1461.39	C-H (CH_2) bending
1383.09	O-H (C-OH) bending

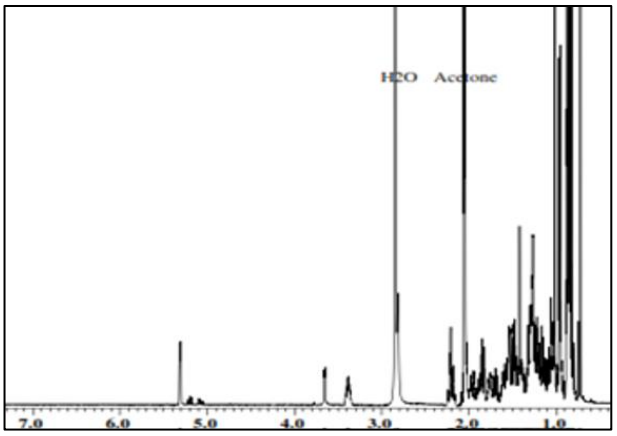

Figure 4. The Spectrum ^{1}H-NMR of β-sitosterol

The ^{13}C-NMR spectrum (Figure 5) shows the appearance of 29 carbon signals. The DEPT-135 spectrum (Figure 6) displays the peaks for the CH_3, and CH while peaks that appear in the 20-40 ppm region indicate the presence of eleven CH_2 groups. Three signals that do not appear in the DEPT 135 spectrum (Figure 6) confirm the three quaternary carbons.

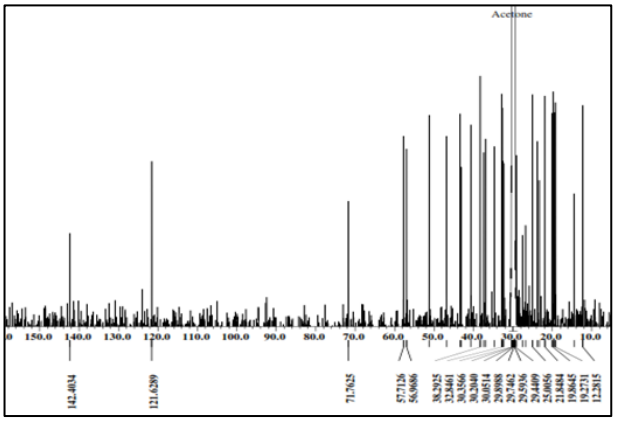

Figure 5. The ^{13}C-NMR of β-sitosterol

The carbon signals at δ 121.61 and 142.4 ppm are assigned to the olefinic carbons (C=C) of C_5 and C_6. The C_3 attached to the β-OH group at δ 71.76 ppm and the signal at δ 12.28 and 19.37 ppm are assigned to the angular methyl carbons of C_{18} and C_{19}, respectively.

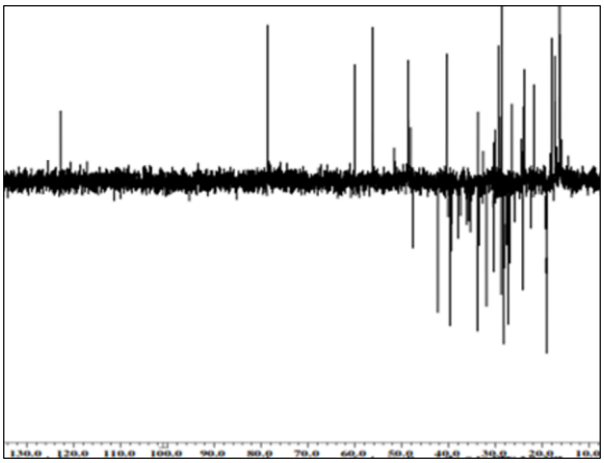

Figure 6. The DEPT 135 of β-sitosterol

The mass molecular ion peak at *m/z* 414 indicates that the compound has a calculated theoretical value for $C_{29}H_{50}O$ as shown in Figure 7. The peak at *m/z* 301 corresponds to [M-113] or the loss of ($-C_8H_{17}$), the peak at *m/z* 273 resembles to [M-141] or loss of aliphatic chain ($-C_{10}H_{21}$), and the peak at *m/z* 396 parallels to [M-18] or loss of hydroxy group. Based on spectroscopy analysis and comparison data from the previous report, the structure of the pure isolated compound is determined as β-sitosterol.

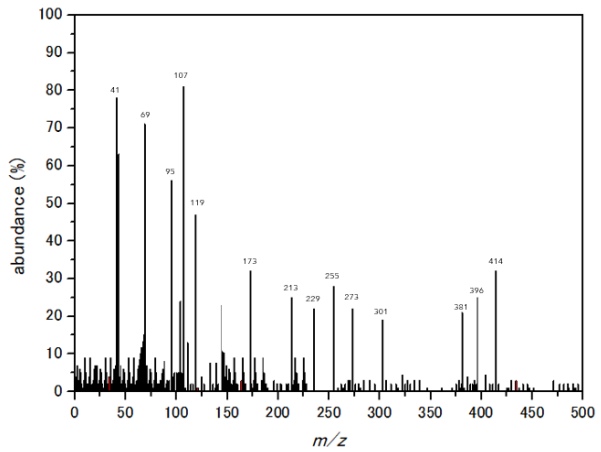

Figure 7. The GC-MS spectrum of β-sitosterol

The chemical shift data in Table 2 show that the NMR spectrum is similar to the β-sitosterol data reported by Patra *et al.* [27]. All the NMR data of β-sitosterol are summarized in Table 2 and modelled in Figure 8.

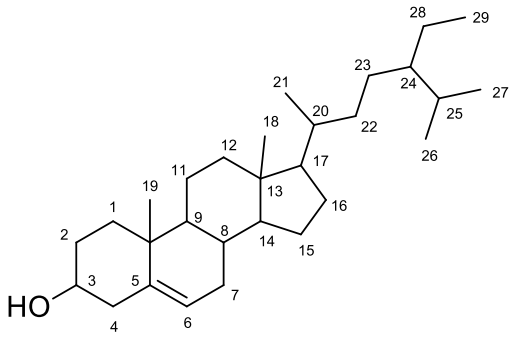

Figure 8. The structure of β-sitosterol

Table 2. The Spectrum ^{1}H-NMR, ^{13}C-NMR, and DEPT 135 of β-sitosterol

No. C	The pure β-sitosterol isolate			Literature [27]		
	^{13}C-NMR (δ, ppm)	DEPT 135	^{1}H-NMR (δ, ppm)	^{13}C-NMR (δ, ppm)	DEPT 135	^{1}H-NMR (δ, ppm)
1	38.29	CH_2		37.28	CH_2	
2	31.69	CH_2	2.3 (*m*)	31.69	CH_2	2.31 (*m*)
3	71.76	CH-OH	3.52 (1H, *m*)	71.82	CH-OH	3.52 (1H, *m*)
4	43.41	CH_2	2.3 (*m*)	42.33	CH_2	2.31 (*m*)
5	142.4	C		140.7	C	
6	121.62	CH	5.38 (1H, *d*)	121.72	CH	5.36 (*d*)
7	32.68	CH_2	2.01	31.69	CH_2	2.08 (*d*)
8	32.84	CH		31.93	CH	
9	51.26	CH		50.17	CH	
10	37.36	C		36.52	C	
11	21.84	CH_2		21.1	CH_2	
12	40.71	CH_2		39.8	CH_2	
13	43.14	C		42.33	C	
14	56.96	CH		56.79	CH	
15	23.79	CH_2		24.37	CH_2	
16	29.02	CH_2		28.25	CH_2	
17	57.71	CH		56.09	CH	
18	12.28	CH_3	0.68 (3H, *s*)	11.86	CH_3	0.68 (3H, *s*)
19	19.27	CH_3		19.4	CH_3	
20	36.99	CH		36.52	CH	
21	19.27	CH_3		18.79	CH_3	
22	34.74	CH_2		33.98	CH_2	
23	26.77	CH_2		26.14	CH_2	
24	46.76	CH		45.88	CH	
25	29.2	CH		28.91	CH	
26	20.15	CH_3	0.84 (3H, *d*)	19.8	CH_3	0.83 (3H, *d*)
27	19.11	CH_3	0.82 (3H, *d*)	18.79	CH_3	0.85 (3H, *d*)
28	23.79	CH_2		23.1	CH_2	
29	12.32	CH_3	0.84 (3H, *d*)	11.99	CH_3	0.86 (3H, *d*)

The crude extract exhibits more inhibition zones than pure isolated of β-sitosterol because the crude extract contains many other bioactive compounds that have not been identified as shown in Figure 9. Based on the zone of inhibition (ZOI) classification, according to Davis and Stout [28], the four strength that correspond to ZOI diameters: > 20 mm are classified as very strong inhibition, 10-20 mm as strong inhibition, 5-10 mm as moderate inhibition and <5 mm is classified as no inhibition. The β-sitosterol had the potential to fight *E. coli* with moderate activity compared to the two positive controls as summarized in Table 3.

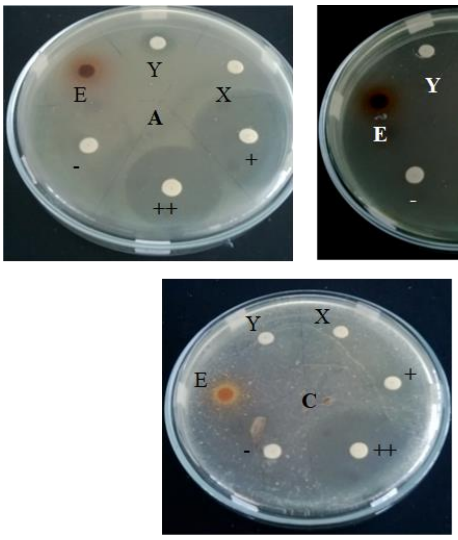

Figure 9. The Antimicrobial activity of β-sitosterol against (**A**) *E. coli*; (**B**) *B. Subtilis* and (**C**) *A. niger*; (E) Crude extract, (Y) β-sitosterol against; (X) Compound X; (+) and (++) positive control; (-) acetone.

Table 3. Antimicrobial activity of β-sitosterol from the root bark of *R. apiculata.*

Symbo	Samples	Concentrations (mg/mL)	Zone of inhibition in mm		
			E. coli	*B. subtilis*	*A. niger*
E	Crude extract	10	16 ± 2	14 ± 1	10 ± 2
Y	β-sitosterol	10	8 ± 2	-	-
+	Chloramphenicol	10	26 ± 3	29 ± 2	17 ± 2
++	Clotrimazole	10	21 ± 1	19 ± 3	24 ± 4
-	Negative control	0	-	-	-

Previous reports have exhibited that β-sitosterol has antibacterial activity against *S. aureus* and *E. coli* [29]. The β-sitosterol inhibited the growth of *E. coli* (14.5 ± 1.84 mm) and *S. aureus* (17.83 ± 0.58 mm) [30]. The differences in the β-sitosterol activity to generate an inhibition zone are presumably dependent on the condition of the antimicrobial assay. The different antimicrobial assay conditions have different sample solvents and microbial test concentrations [31]. The β-sitosterol has been registered as a non-toxic and safe supplement. β-sitosterol has been registered as a non-toxic and safe supplement. It has potential bioactivities in several applications, including antibacterial activity.

4. Conclusion

This study reported the isolation and structure elucidation of β-sitosterol from the root bark of Bakau Minyak from Lempasing coastal, South Lampung. This study supported the use of Bakau Minyak as a traditional medicine to treat several diseases such as infections by *E. coli*. The antibacterial activity of β-sitosterol from the root bark of *R. apiculata* exhibited potential bioactivity to be developed as a lead compound for bacterial infections. The high potency of β-sitosterol and its analogs in treating various diseases classifies this compound as an essential drug in the future.

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